

Developing a multi-component immune model for evaluating the risk of respiratory illness in athletes

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ABSTRACT

Clinical and laboratory identification of the underlying risk of respiratory illness in athletes has proved problematic. The aim of this study was to determine whether clinical data, combined with immune responses to standardised exercise protocols and genetic cytokine polymorphism status, could identify the risk of respiratory illness (symptoms) in a cohort of highly-trained athletes. Male endurance athletes ($n=16$; VO_{2max} 66.5 ± 5.1 mL.kg⁻¹.min⁻¹) underwent a clinical evaluation of known risk factors by a physician and comprehensive laboratory analysis of immune responses both at rest and after two cycling ergometer tests: 60 min at 65% VO_{2max} (LONG); and 6 x 3 min intervals at 90% VO_{2max} (INTENSE). Blood tests were performed to determine Epstein Barr virus (EBV) status and DNA was genotyped for a panel of cytokine gene polymorphisms. Saliva was collected for measurement of IgA and detection of EBV DNA. Athletes were then followed for 9 months for self-reported episodes of respiratory illness, with confirmation of the underlying cause by a sports physician. There were no associations with risk of respiratory illness identified for any parameter assessed in the clinical evaluations. The laboratory parameters associated with an increased risk of respiratory illnesses in highly-trained athletes were cytokine gene polymorphisms for the high expression of IL-6 and IFN- γ ; expression of EBV-DNA in saliva; and low levels of salivary IgA concentration. A genetic risk score was de-

veloped for the cumulative number of minor alleles for the cytokines evaluated. Athletes prone to recurrent respiratory illness were more likely to have immune disturbances that allow viral reactivation, and a genetic predisposition to pro-inflammatory cytokine responses to intense exercise.

KEYWORDS: exercise, athletes, respiratory infections, inflammation

INTRODUCTION

Upper respiratory illness is the most common reason for non-injury related presentation in sports medicine, accounting for 35–65% of illness presentations in elite athletes in training and competition (12, 14, 41). Recurrent respiratory illness can have a negative impact on the health and performance of athletes undertaking high levels of strenuous exercise, and interferes with training and ability to compete in international competitions in up to 10% of athletes (2, 48, 52). The majority of athletes have a similar incidence of upper respiratory illness to the general population (15), but a small proportion (5–7%) experience recurrent episodes of upper respiratory symptoms (URS) at significantly higher rates. The incidence of URS increases during periods of intense training, in association with increases in training load (32, 38) and around competitions (41, 44, 55). Identifying athletes at risk of recurrent URS allows adoption of preventative strategies based on relevant clinical, training and lifestyle modifications.

The common symptoms associated with upper respiratory illness include a sore throat, headache, fatigue, runny nose and/or watery eyes. The cause of URS in athletes is often unknown as pathology testing is rarely undertaken and physicians may not be available to undertake comprehensive clinical assessments in research studies. Pathology investigations have identified infections as a cause of the symptoms in only 30–40% of high-performance athletes studied (6, 53). A higher frequency has been observed in recreational athletes (28). Bacterial respiratory infections are uncommon in elite athletes (25) and the majority of identified infections are common res-

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piratory viruses found in the general population (6, 15, 29, 49, 53). While transient exercise-induced immune suppression can increase susceptibility to infection (58), not all episodes of URS have an infective aetiology (6, 49) and susceptibility is influenced by other lifestyle and environmental factors (29, 38, 55).

Non-infective inflammatory causes of URS include allergic responses to aeroallergens, asthma, and trauma to respiratory epithelial membranes, particularly in athletes who experience drying of the airways related to increased expired ventilation or cold air exposure (27). Undiagnosed or inappropriately treated asthma and/or allergy are common findings in clinical assessments of elite athletes experiencing recurrent URS (6, 49). Inhaled allergens can affect approximately 40% of athletes (6, 49) with 20-40% having rhinoconjunctivitis (6, 35, 56) that responds to treatment with topical medications (7, 34). Psychological stress, sleep disturbances and dietary deficiencies have also been associated with increased URS (30, 38).

There has been extensive examination of changes in immune parameters with exercise, but only a limited number have included assessment of URS in the study design (27, 29, 33). Many of the conclusions that both high levels of exercise intensity and sedentary behaviours are associated with susceptibility to URS have been inferred from the changes in immune parameters. These changes have the potential to leave a person at increased risk of infection.

The measurement of secretory IgA in saliva has shown consistent associations with URS in athletic populations (58). Secretory IgA is an important component of protection against infections at mucosal surfaces, together with integrity of the epithelial barrier and regulatory T-lymphoid cells. Low levels of salivary IgA, decreased IgA secretion rates, and a decline in salivary IgA concentration over a training period have been associated with a higher risk of URS (25, 26, 33, 44). Moderate exercise, as opposed to intense exercise, can increase salivary IgA and has been used to modify training in athletes at risk of or with a history of recurrent URS (23, 37). A recent study of tear fluid secretory IgA (SIgA) has also shown lower levels of SIgA and excretion rates in tears but not in saliva with an increase in the risk of upper respiratory tract infections in athletes (29).

Illness-prone athletes can have altered/adverse cytokine responses to standardised intense treadmill exercise protocols in comparison with healthy athletes (11) and a genetic predisposition to pro-inflammatory cytokine responses (5). The cytokine responses to the exercise included lower resting levels of IL-8, IL-10 and IL-1ra in illness-prone athletes, lower levels of IL-10 and IL-1ra but higher levels of IL-6 post exercise, collectively indicating impaired inflammatory cytokine regulation in illness-prone athletes (11). A study of cytokine gene polymorphisms identified a trend for a high-expression genotype for IL-6 in illness-prone athletes and a high-expression genotype for IL-2 associated with a decreased likelihood of recurrent URS in a cohort of 170 elite athletes (5). Two studies differ on the impact of IL-10 genotypes on risk of URS, with one showing no impact (5) and the other indicating an increased risk with the high-expression IL-10 genotype (61).

Studies have examined the effectiveness of various biomarkers to identify athletes at risk of URS, based on the premise that transient immune alterations after exercise provide a window of opportunity for infections. While fitter adults are less likely to experience URS than sedentary individuals (4), this paradigm does not hold true for the 7-10% of elite athletes who experience recurrent URS (15, 24, 30). There appears to be a threshold of training load that puts athletes at increased risk of URS. Longitudinal studies have identified the impacts of intense training (24, 39) on decreased concentration or excretion rates of salivary IgA and an increased risk of URS (58).

Epstein Barr virus (EBV) infection is a common presentation in elite athletes and viral reactivation of EBV is a common finding in research settings (22-50%) in athletes experiencing recurrent URS (8, 60). Expression of EBV DNA in saliva is associated with a prior reduction in salivary IgA levels and subsequent appearance of URS (28, 60). IgA plays a major role in controlling viral reactivation and low levels prior to the appearance of EBV viral DNA indicate that salivary IgA could be used as a surrogate marker for increased risk of viral reactivation and the associated inflammation that occurs from the immune response to the viral particles shed in the respiratory tract (20). Midkine is a constituent of the mucosal innate immune system with potent bactericidal and fungicidal properties and plays a role in inflammatory processes at mucosal surfaces (19). Traditional biomarkers of innate immunity, such as lactoferrin and lysozyme, have also shown associations with URS in athletes (59). Midkine concentration has not been assessed previously in relation to responses to intense exercise and was included in this study as a potential new marker of risk for URS in elite athletes.

The aim of this study was to determine whether clinical data, combined with immune responses to standardised exercise protocols and genetic cytokine polymorphism status, can be used to identify the risk of respiratory illness (symptoms) and associated fatigue in elite athletes. This study assessed selected clinical and laboratory parameters, known to be associated with URS, in a prospective study to identify athletes at risk of a high incidence of URS. The study design included a clinical assessment for known causes of respiratory infections and airway inflammation, laboratory tests for genetic predisposition to pro-inflammatory responses and EBV status, and immune responses to short-intense and longer-endurance exercise protocols. The study involved highly-trained male cyclists who were monitored daily for nine months and then classified as illness-prone or healthy based on their reported episodes of URS during the study period. The clinical and laboratory parameters were then assessed for their effectiveness in a predictive model.

MATERIALS AND METHODS

Study Design

The study examined clinical and laboratory measures associated with highly-trained endurance athletes who experience recurrent episodes of upper respiratory tract illness due to infection and/or inflammation.

Prior to commencement of the investigations each athlete underwent a comprehensive clinical examination with a focus on a history of known causes of airway infection and inflammation, including asthma, allergy, and common respiratory infections. A full blood count (FBC) was performed prior to undertaking performance testing to exclude underlying infections and potential exclusion illnesses. Blood serology tests were performed to determine Epstein Barr virus (EBV) status and DNA was examined for cytokine gene polymorphisms. Saliva was collected prior to the VO_2max test to exclude any subject with IgA-deficiency.

Sixteen endurance-trained male cyclists were recruited to this study and prospectively followed for 9 months using a web-based daily reporting of training and illness symptoms. The type, severity and duration of illness were quantified using the AIS Athlete Illness Questionnaire (17). Each episode of illness was followed up by a physician and included completion of the Common Cold Questionnaire (46)

Each athlete completed an initial performance assessment including a VO_2max test and two subsequent cycle ergometer tests, with each test at least 7 days apart, to assess a multi-component immunological response to the exercise protocols. The VO_2max test was performed prior to commencing the study and the Long and Intense exercise tests were completed in a randomised order in the first two weeks of the study. These included plasma cytokines (IL-1ra, IL-4, IL-6, IL-8, IL-10, IL-17a, interferon- γ (IFN- γ)), C-reactive protein (CRP), salivary IgA and salivary EBV-DNA at several time points before and after the exercise tests (pre-exercise, and immediately, 1hr and 24 hr post-exercise). Saliva and blood samples were processed for storage under appropriate conditions for analysis as single batches at the conclusion of the study to reduce assay variability.

Exercise Testing - VO_2max test

At the initial visit, each athlete completed a VO_2max test. The maximal aerobic capacity (VO_2max) of each athlete was assessed by an incremental exercise test to volitional exhaustion on a Lode Excalibur cycle ergometer (Load B.V. Groningen, The Netherlands) and open-circuit indirect calorimetry system (Australian Institute of Sport, Bruce, ACT, Australia) as described previously (11). Athletes commenced at 100W with an increase of 25W every 3 min until volitional exhaustion. VO_2 , heart rate and blood lactate were monitored throughout the test by standard techniques.

Exercise Protocols – Long and Intense

Each athlete completed two cycling ergometer tests in a randomised and counterbalanced order, with tests separated by a minimum of 7 days. Testing was conducted between 8-10am. Exercise testing was only undertaken if athletes were free of symptoms of illness on the scheduled day of testing and in the previous 3 days. The test protocols were: (i) 60 min at 65% VO_2 max (LONG); and (ii) 6 x 3 min intervals at 90% VO_2 max with 90 seconds of active recovery between each repetition (INTENSE). Athletes completed a 5 min warm-up on the cycle ergometer involving 3 min at a self-selected power output (range 100-150 W) then 4 x 15 sec intervals at the power output of the designated exercise intensity followed by 15 sec

of self-selected active recovery. Heart rate was recorded continuously during the trial, and subjects assessed their effort using the Borg Scale 1-10 rating of perceived exertion. Whole blood lactate concentration was monitored as a measure of exertion using the Lactate Pro analyser (Arkay KDK, Japan) with 5 μl blood drawn from the earlobe or fingertip.

Laboratory Methodology

Blood Collection

Blood was collected prior to the VO_2max tests and 24 h after the exercise protocols from a superficial fore arm vein by standard venepuncture techniques. An intravenous cannula was inserted prior to commencement of each cycle ergometer exercise test to allow multiple timed blood collections, prior to and immediately after and at 1 h post exercise. Samples were collected into K_3EDTA and clot activator serum separation tubes (Greiner Bio-one, Frickenhausen, Germany).

Full Blood Count

A full blood count (FBC), including a white blood cell (WBC) differential for enumeration of neutrophil, lymphocyte and monocyte, basophil and eosinophil populations, was performed on whole blood samples within one hour of sample collection using a Sysmex XT-2000i Counter (Sysmex Corporation, Japan).

EBV serology

Serum IgM antibodies to EBV viral capsid antigen and IgG antibodies to EBV nuclear antigen were measured as previously described (8) with commercial enzyme-linked immunosorbent assay (ELISA) kits (panbio; Inverness Medical Innovations, Sinnamon Park, QLD, Australia) using a BEP2000 Advance Analyser (Siemens, Munich, Germany). All samples were analysed in a single batch to avoid inter-assay variation.

C-Reactive Protein

Serum CRP concentrations were determined using an Immulite 1000 solid phase chemiluminescent immunometric assay system (Siemens Healthcare Diagnostics, Flanders, NJ, USA) and commercially available assay kits (Diagnostics Products Corporation, CA, USA). All samples were analysed in a single batch to avoid inter-assay variation. The population reference range for serum CRP concentration was <3 mg/L.

Cytokine Concentrations

Blood samples were collected directly into K_3EDTA tubes (Greiner Bio-one; Frickenhausen, Germany) and plasma separated by centrifugation at 800g for 5 min and stored frozen at -80 °C until analysed. Plasma concentrations of each cytokine were determined simultaneously using a Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA) and custom manufactured Multiplex Cytokine Kits (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA) as previously described (11). The instrument was standardised with Bio-Plex Pro Human Cytokine Standard 27-Plex Group 1, Lot number 50295100 (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA).

Cytokine Polymorphisms

Nucleic acids were extracted from whole blood cells collected in K_3EDTA tubes using the QIAamp Blood Mini Kit (QIA-

GEN GmbH, Hilden, Germany). Extracted RNA was stored in Qiagen RB Sample Tubes (QIAGEN GmbH, Hilden, Germany) at -80°C until assayed as a single batch. Assays were completed in accordance with manufacturer's guidelines using a 7500 Real Time PCR System (PE Applied Biosystems, Foster City, USA) as previously described (5). Automatic classification of samples as homozygous (for either allele) or heterozygous was undertaken using the SDS 7500 System Software Version 1.4 (PE Applied Biosystems). The cytokine polymorphisms assessed are listed in Table 8. The classification of each polymorphism was determined from the NCBI dsSNP database (available at www.ncbi.nlm.nih.gov/SNP/).

Midkine

Midkine concentrations were measured using a commercial enzyme-linked immunosorbent assay (EBV) (Cellmid Limited, Sydney, Australia).

Saliva Collection

Saliva samples were collected passively using four commercial eye spear swabs (CoreSurgical, UK) 10 min prior to the VO_2max test, immediately prior to each exercise test, immediately after completion of each test, and at the 1 h and 24 h recovery time points. The athletes were not fasted. The eye spear swabs have been confirmed as a suitable collection method for analysis of salivary IgA (54).

Salivary IgA Concentration

The concentration of IgA was measured in each saliva sample by an in-house ELISA method as described previously (22). The between-run coefficient of variation for the internal control was 11%.

Salivary EBV-DNA

EBV viral excretion in saliva was detected using a quantitative real-time polymerase chain reaction assay. DNA was isolated from saliva samples using a QIAamp DNA Mini Kit (QIAGEN, GmbH, Hilden, Germany). Commercially available EBV-specific primers and probes (Qubit dsNA BR Assay kit, Invitrogen, Carlsbad, California, USA) were used in the real time PCR to amplify a region of the BALF5 gene, as described previously (8), using a Viia-7 rtPCR System (PE Applied Biosystems, Foster City, USA) detection system.

Statistical Analysis

All statistical analyses were performed using SAS v9.4 (SAS Institute, Cary, North Carolina, USA). Given the multiple potential outcomes measured, the Bonferroni method of adjusting for multiple comparisons was adopted. The significance level was set at $p < 0.01$.

Differences between the illness-prone and healthy athletes in age, weight, fitness level, clinical history, and pre-exercise salivary IgA concentration were assessed using 2-sample t-tests or Pearson chi-square tests. Salivary IgA data were summarised at each time point using medians (with 95% confidence intervals). Within-subject differences in median salivary IgA were assessed using the sign-rank test.

For each of the cytokine SNPs the major/minor alleles in a Caucasian population were chosen using the NCBI dbSNP

database (<http://www.ncbi.nlm.nih.gov/SNP/>), as the participants were Caucasian. The NCBI database for IFN- γ has T as the major allele in Caucasian populations. In both this study and a previous study (5) the A allele appears to be the major IFN- γ SNP in the Australian population. The impact of coding the major and minor allele for IFN- γ was assessed both ways for this study. The impact of coding A as the major allele would lead to increased inflammation for the (minor) T allele. The classification was also checked both ways in the genetic risk score analysis.

The distribution of each cytokine SNP was assessed for Hardy-Weinberg equilibrium prior to analysis. Genotypes and their association with the dichotomous outcome (illness prone or healthy, based on the number of URS during the study period) were analysed using two methods. First, individual genotyped SNPs were coded as 0, 1, or 2, representing a subject's dosage (number of copies) of the minor allele. Association with the outcome was analysed with a chi-square test of general association. Secondly, a composite genetic risk score (GRS) was generated representing the total number of minor alleles across all candidate cytokine SNPs in each individual. In this case association with the outcome was analysed using a t-test for the difference in mean genetic risk score.

As the dataset sample size was small, exact logistic regression was used to analyse the outcome against each SNP under an additive model. Estimates showing the odds ratio for the outcome with 95% confidence intervals are presented (Table 10). P-values from the exact tests are presented. As outlined above, given the number of cytokines tested, we altered the threshold for significance to $p < 0.01$.

The distribution of concentrations of salivary IgA (Figure 2) and midkine (Figure 3) are presented as box and whisker plots at each time point for the Intense and Long exercise protocols. The bars in the box plot represents the 25th, 50th, 75th percentiles, the circle within the boxes (joined by dashed lines) represents the mean concentration. Circles outside the box illustrate points that exceeded 1.5 times the interquartile range above the 75th percentile.

RESULTS

Study cohort

Complete clinical and laboratory data sets were obtained from 16 male athletes. The athletes were triathletes ($n=4$), cyclists ($n=11$) and a cross country skier ($n=1$) whose training included long distance cycling. Eight athletes competed at national or international level and the other eight at state or club level. The number of clinician-verified URS episodes during the study was used to classify the subjects as illness prone (>3 episodes). Only 4 subjects met the definition for illness-prone, which has limited the power to detect differences between the illness-prone and healthy athletes.

The physical characteristics of the study cohort were: age 32.5 ± 8.1 y; body mass 73.9 ± 7.9 kg; VO_2max 4.9 ± 0.6 L.min⁻¹; VO_2max 66.5 ± 5.1 mL.kg⁻¹.min⁻¹; peak power 411 ± 46 W; 5.6 ± 0.5 W.kg⁻¹; mean \pm SD. The mean duration of training

Table 1. Demographics of the athletes in the illness-prone (n=4) and healthy (n=12) athlete groupings and comparison between groups (mean (SD)).

Variable	Illness-prone (n=4)	Healthy (n=12)	p-value
Age (years)	28.6 (7.9)	33.8 (8.1)	0.28
Body mass (kg)	70.6 (6.0)	74.5 (8.3)	0.41
VO ₂ max (L/min)	5.0 (0.3)	4.9 (0.6)	0.67
(ml/kg/min)	69.6 (3.6)	65.5 (5.2)	0.12
Peak Power (W)	431 (38)	404 (48)	0.32
(W/kg)	5.5 (0.5)	6.0 (0.5)	0.10

for the group was 14 ± 5 h per week. There were no differences between the illness-prone and healthy athletes for age, weight, or fitness level determined by VO₂max performance

was unremarkable (Table 2). The results of the full blood count testing indicated the subjects were clinically healthy and showed no signs of infections or inflammation at the time of exercise testing. Six athletes (38%) had a history of asthma but only one athlete was currently being treated for asthma. Eight athletes (50%) had a history of allergy, with five having allergic rhino-conjunctivitis and 3 recording other allergies. There was no difference in the distribution of a history of any allergy ($p=0.25$), allergic rhino-conjunctivitis ($p=0.12$), asthma ($p=0.55$), or a combined history of asthma and/or allergy ($p=0.07$) between the illness-prone and healthy athletes (Table 2).

Upper Respiratory Tract Symptom Episodes

The 16 athletes had evenly distributed episodes of upper respiratory tract symptoms (URS) during the 9 month prospective study (southern hemisphere spring, summer, autumn). There were no episodes in 3 athletes (19%); 9 athletes had 1-2 episodes (56%); and 4 athletes had 4-5 episodes (25%). On

Table 2. Reported episodes of URS during the study by each athlete, their clinical history of URTI, asthma and allergy and EBV serology status at commencement of the study, and detection of EBV-DNA in saliva samples collected during the exercise protocols. Blank spaces indicate a negative result. RC indicates the allergy was rhino-conjunctivitis. URS upper respiratory symptom, URTI upper respiratory tract illness, EBV Epstein Barr virus, DNA deoxyribonucleic acid, PRE pre-exercise, immediately POST and 24hr post-exercise. No sample was collected 24hr post Intense protocol for ID #9.

ID	Number of URS episodes during study	History of URTI in prior 12mth	History of Asthma	History of Allergy	EBV Serology Status	EBV-DNA in saliva LONG Protocol				EBV-DNA in saliva INTENSE Protocol		
						PRE VO ₂ max test	PRE test	POST test	24HR Post	PRE test	POST test	24HR Post
1	0	3			Positive	Positive	Positive	Positive				
2	0	2		Yes/ RC	Positive				Positive	Positive	Positive	
3	0	1	Yes		Positive							
4	1	1		Yes	Positive							
5	1	3	Current	Yes/ RC	Positive				Positive	Positive		
6	1	2		Yes/ RC	Positive		Positive	Positive				
7	1	1		Yes/ RC	Positive		Positive	Positive	Positive		Positive	
8	2	3	Yes	Yes/ RC	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
9	2	2			Positive							Positive no data
10	2	1	Yes		Positive	Positive	Positive					
11	2	0			Positive	Positive	Positive	Positive	Positive			Positive
12	2	2	Yes	Yes	Positive	Positive				Positive	Positive	
13	4	15			Positive		Positive					
14	4	2			Positive	Positive	Positive	Positive	Positive			
15	4	2			Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
16	5	0	Yes	Yes	Negative							

(Table 1). After adjustment for body mass there was a trend for illness-prone athletes to have a higher relative VO₂ max (ml.kg/min) ($p=0.12$) and relative peak power (W/kg) ($p=0.10$) than the healthy athletes (Table 1).

Clinical History

Assessment of the clinical history and clinical interview data

average the symptoms lasted 4 days during each episode. There was no significant difference in the distribution of the number of URTI episodes reported in the previous 12 months ($p=0.25$) between the illness-prone and healthy athletes (Table 2). The self-reported history of URTI in the 12 months prior to the study did not match the number of URS episodes reported during the 9-month study period (Table 2).

Table 3. Number of URS episodes reported by the 15 EBV-seropositive athletes and the number and percentage of saliva samples with EBV-DNA detected in these athletes. No subject reported 3 URS episodes during the study.

Number of URS episodes during the study in the 15 EBV seropositive athletes	Number of athletes	Number of Pre-exercise saliva samples with EBV-DNA detected	Percentage of Pre-exercise samples with EBV-DNA detected	Number of samples from all exercise tests with EBV-DNA detected	Percentage of samples from all exercise tests with EBV-DNA detected
0	3	3/9	33%	6/21	29%
1	4	3/12	25%	7/28	25%
2	5	8/15	53%	17/34	50%
4	3	6/9	67%	12/21	57%

EBV Serology

All athletes were negative for EBV IgM serology at the start of the study, indicating no athlete had current infectious mononucleosis (glandular fever). Based on the IgG serology, 15 athletes were seropositive for prior EBV infection (94%). One athlete (ID#16) was seronegative, had no EBV-DNA detected in any saliva sample, but notably the highest incidence of URS during the study (Table 2).

Table 4. The pre-exercise test (PRE) salivary (Sal) IgA concentrations (mg/L) and mean of the three pre-exercise test results for each athlete compared to the number of reported episodes of URS and the predictive Risk category of the salivary IgA concentration. Samples were classified as Higher Risk (H) of URS for IgA <40mg/L, Lower Risk (L) for IgA > 60mg/L and Moderate Risk (M) for IgA between 40-60 mg/L for the initial VO₂max test and for the average of the three pre-exercise test (3 x PRE) saliva samples.

Subject ID	URS Episodes in study	VO ₂ max PRE Sal IgA	Intense PRE Sal IgA	Long PRE Sal IgA	Mean of 3 x PRE Sal IgA	Risk for VO ₂ max PRE	Risk for Mean of 3 x PRE
1	0	58	33	26	39	M	H
2	0	94	77	64	78	L	L
3	0	67	22	50	46	L	M
4	1	107	58	48	71	L	L
5	1	62	32	90	61	L	L
6	1	48	43	67	53	M	M
7	1	41	64	68	41	M	M
8	2	50	42	32	41	M	M
9	2	38	40	44	41	H	M
10	2	102	60	100	87	L	L
11	2	127	72	21	73	L	L
12	2	31	48	59	46	H	M
13	4	38	35	24	32	H	H
14	4	62	53	39	51	L	M
15	4	135	71	80	95	L	L
16	5	37	28	31	32	H	H
Mean±SD		69 ± 34	49 ± 17	53 ± 24	55 ± 20		

Table 5: The distribution and median concentrations of the average of the three pre-exercise salivary IgA concentration (mg/L) in the illness-prone and healthy athletes and the differences between the groups.

Pre-exercise salivary IgA concentration	Class	Illness-prone (n=4)	Healthy (n=12)	Total (n=16)	Difference p value
Lower Risk	>40mg/L	2 (15%)	11 (85%)	13 (81%)	0.06
Higher Risk	<40mg/L	2 (67%)	1 (33%)	3 (9%)	
Median IgA	mg/L	42	55		0.44
Min, Max	mg/L	32, 95	39, 87		

Table 6. Salivary IgA concentrations (mg/L) and 95% confidence intervals (CI) for all athletes undertaking the Intense and Long Exercise Protocols at each collection time

Saliva Collection Time	Intense Protocol Salivary IgA median (95% CI)	Long Protocol Salivary IgA median (95% CI)
Pre-exercise	45 (35, 64)	49 (32, 69)
Post-exercise	53 (38, 68)	54 (46, 64)
1 h Post	43 (33, 55)	45 (37, 78)
24 h Post	39 (28, 63)	38 (27, 56)

Table 7: Change in salivary IgA concentration (mg/L) both between- and within-subject across the time points in the Intense and Long Exercise Protocols. The sign of the change indicates the direction of median change in percentage concentration: positive is an increase; negative is a decrease.

Difference	Intense Protocol median (95% CI)	Sign Rank p value	Long Protocol median (95% CI)	Sign Rank p value
Between-subject				
Pre - Post	+0.3 (-10, +22)	0.72	-0.5 (-16, +15)	0.94
Post - 1 h	-6.9 (-21, +11)	0.21	-6.8 (-12, +25)	1.00
Post - 24 h	-5.6 (-28, -0.4)	0.03	-12 (-20, +24)	0.60
24 h - Pre	-2.1 (-19, +3)	0.42	-4.2 (-12, +12)	0.74
Within-subject				
Pre - Post	+0.2 (-20, +43)	0.56	-0.8 (-25, +61)	0.53
Post - 1 Hour	-13.2 (-28, +47)	0.56	-18 (-26, +46)	0.90
Post - 24 Hour	-9.4 (-50, -0.6)	0.05	-27 (-35, +37)	0.63
24 Hour - Pre	-4.1 (-37, +9)	0.42	-9.6 (-27, +27)	0.78

EBV viral DNA

EBV-DNA was detected more frequently in saliva samples prior to the VO₂max test, and also pre and post the two exercise protocols in athletes with a higher number of episodes of URS during the study (Table 2). The percentage of saliva samples positive for EBV-DNA in the three PRE-exercise saliva samples tended to increase with incidence of URS (p=0.14) from 33% to 67% (Table 3). Saliva samples collected immediately post and at 24h post the exercise protocols were tested for EBV-DNA (Table 3) and showed the same trend of higher detection rates in athletes with a higher number of episodes of URS (p=0.06), increasing from 24% to 52% (Table 3).

Salivary IgA

All athletes had detectable levels of IgA in all saliva samples, confirming there were no IgA-deficient subjects. Using previously established URS risk cut-off levels for swimmers (26) the athletes were classified for each saliva sample as 'Higher Risk' if salivary IgA was <40 mg.L⁻¹; 'Moderate Risk' if between 40-60 mg.L⁻¹; or at a 'Lower Risk' if >60 mg.L⁻¹ (Table 4). Low levels of salivary IgA (<40 mg.L⁻¹) were more common in the PRE VO₂max test samples for athletes reporting a higher number of URS episodes during the study (Table 4). The average of the three pre-exercise test salivary IgA concentrations was used to estimate a typical within-subject salivary IgA resting level as this takes into account the large variability within-subjects (13). The distribution of the average of the three resting pre-exercise salivary IgA levels approached significance (p=0.06) with a higher proportion of lower levels of salivary IgA (<40mg/L) in the illness-prone athletes (Table 5). The differences in the median concentrations of the average pre-exercise salivary IgA between the illness-prone and healthy groups was lower in the illness-prone group (Table 5) but not significantly different to the healthy athlete group (p=0.44).

Salivary IgA response to exercise

Salivary IgA responses to the Intense and Long exercise protocols showed variability between the athletes. To normalise the response the changes were expressed as a percentage change relative to the resting pre-exercise level for each athlete (Figure 1). The median changes in the salivary IgA concentrations for all athletes (Table 6, Figure 2), the percentage changes between time points for all athletes (Table 7) and the within-person difference (change) in median IgA levels (Table 7) immediately post exercise were not

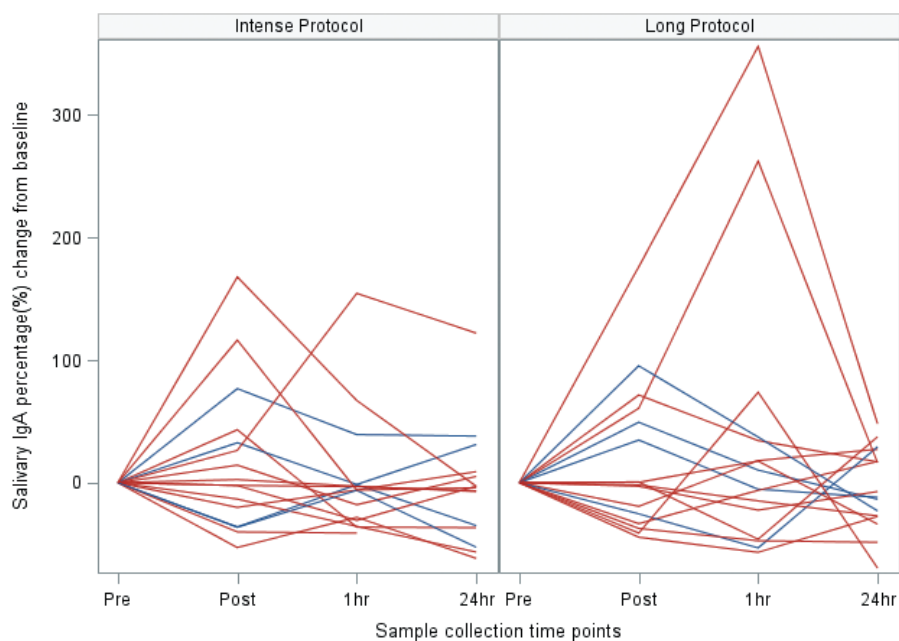


Figure 1: Salivary IgA responses to Long and Intense exercise protocols for each athlete expressed as a percentage change from the resting pre-exercise level for individual athletes. Individuals with more than 3 URS episodes are coded as blue.

significantly different from the baseline pre-exercise levels for both protocols. Differences in median salivary IgA concentrations immediately post to 1h post exercise were not significant for either protocol in any measure (Table 7).

There was evidence of a significant difference at 24 h after the Intense protocol compared to the immediate post (median difference of 8 mg.L^{-1} , $p=0.03$) although this did not remain significant when adjusted for multiple comparisons using the Bonferroni method (Table 7). The percentage change in salivary IgA levels for individual athletes from immediate post to 24 h post in the Intense protocol showed the same trend ($p=0.05$; median change of 9%) although this did not reach

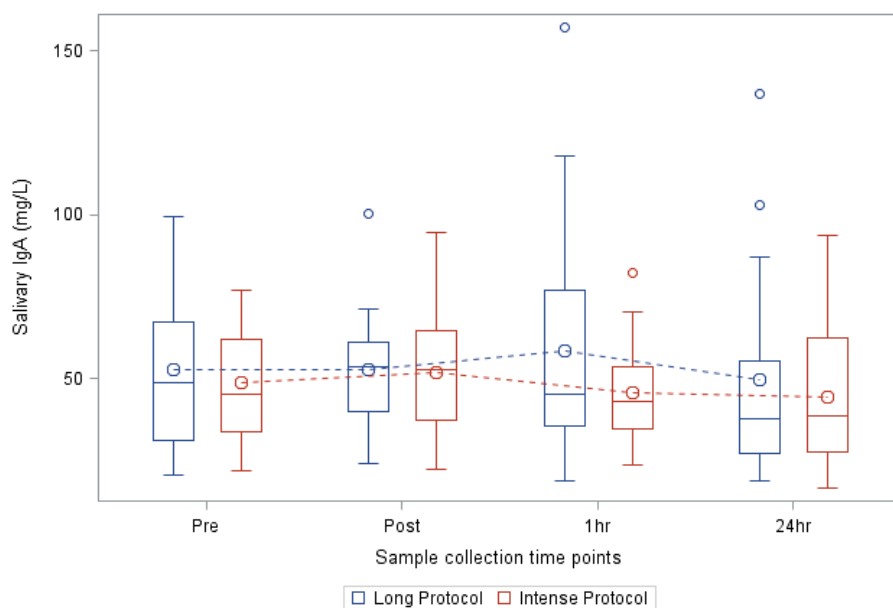


Figure 2: Distribution (median and quartiles in box-plots, mean in circles) of salivary IgA (mg/L) at each time point for the Long (Blue) and Intense (Red) exercise protocols.

our significance threshold of 0.01. There were no significant differences between the 24h post and the pre-exercise test level for either protocol using any of the measures (Tables 7).

CRP and Cytokine Concentrations

There were no elevated CRP levels above the population reference range for any pre-exercise test blood sample. The CRP levels did not alter significantly during either exercise protocol. The pre-exercise test CRP levels did not correlate with the number of episodes of URS. Most of the cytokines analysed using the Multiplex technology were either not detected (IFN- γ) in any sample or detected in only a subset of the athletes (IL-1ra, IL-10, IL-4, IL-6). All athletes had detectable levels of IL-17a and IL-8 on most occasions. As a result there was insufficient power to show any statistical patterns in the cytokine responses to exercise.

Cytokine Genotype SNPs

The distribution of each cytokine SNP polymorphism in the study cohort is provided in Table 8. The exact logistic regression showed there was no clear association between the number of minor alleles for individual SNPs and having three or more URS episodes during the study (Table 9). The exception was a trend for an association with IL-6 ($p=0.06$) and IFN- γ ($p=0.01$). The genetic risk score indicates a potential for an accumulative effect of the number of minor alleles ($p=0.03$) although this did not meet our significance level of $p=0.01$.

The odds ratios (OR) for being illness-prone are provided for each SNP in Table 10. There is some evidence of a cumulative effect of increasing genetic risk score on the odds of three or more illness bouts during the study period (OR=0.49; 95% CI: 0.15,0.98; $p=0.04$) but this was not significant at the 1% threshold. The possible association with IFN- γ minor alleles ($p=0.05$) was also not significant at the 1% level.

Midkine response to exercise

There was a significant increase in midkine concentrations immediately post exercise for both exercise protocols (Table 11, Figure 3). The Midkine concentrations fell below pre-exercise levels at 1 h post but returned to pre-exercise concentrations by 24 h post-exercise.

DISCUSSION

A more effective means of identifying the risk of illness would assist clinicians in

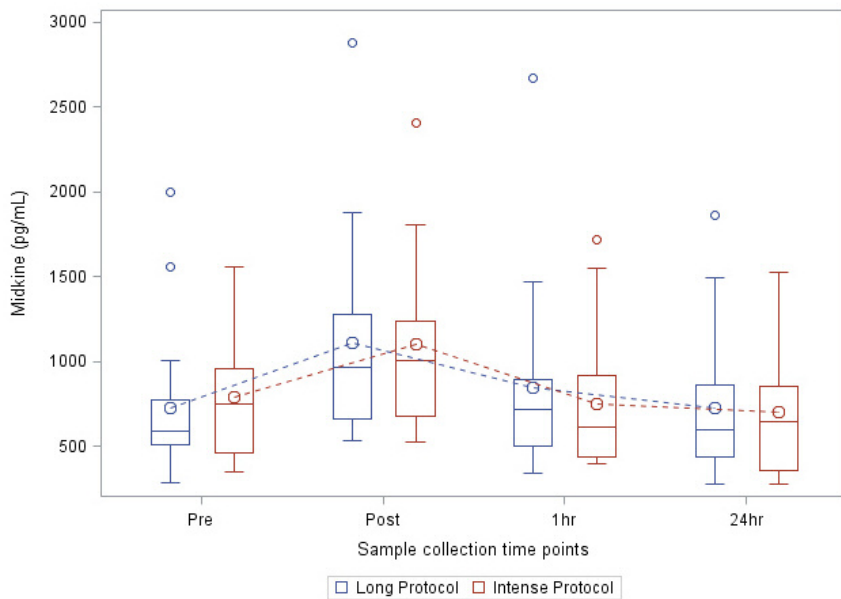


Figure 3: Distribution (median and quartiles in box-plots, mean in circles) of midkine (pg/ml) at each time point for the Long (Blue) and Intense (Red) exercise protocols.

Table 8. Distribution of cytokine polymorphisms in the cohort of athletes (n=16). The minor allele homozygote is identified by grey shading. The NCBI reference number, the polymorphism substitution and the impact of the minor allele on cytokine production are provided. The cytokines have been classified as having pro- (PRO), anti-inflammatory (ANTI) and regulatory (REG) functions.

Cytokine	Poly-morphism (ancestral > replacement minor allele)	SNP reference number (rs number NCBI)	Impact on cytokine production of allele substitution	Number &% of SNPs in the whole cohort (high/moderate/low expression) (NCBI minor allele homozygote)		
				high	moderate	low
PRO						
IL-6	G>C	1800795	decreased expression	GG 6 38%	GC 5 31%	CC 5 31%
IL-8	A>T	4073	increased expression	TT 5 31%	AT 7 44%	AA 4 25%
IL-17a	G>A	1974226	?increased expression	AA 1 6%	AG 4 25%	GG 11 69%
IL-17a	A>G	2275913	decreased expression	AA 1 6%	AG 8 50%	GG 7 44%
ANTI						
IL-10	A>G	1800896	decreased expression	GG 0 0%	AG 4 25%	AA 12 75%
IL-1ra	T>C	419598	decreased expression	TT 8 50%	TC 7 44%	CC 1 6%
REG						
IL-4	C>T	2243250	increased expression	TT 0 0%	CT 5 31%	CC 11 69%
IFN- γ	T>A	2430561	decreased expression	TT 2 13%	TA 10 63%	AA 4 25%

Note: in this study the minor allele for IFN- γ was classified as T and would increase the production of IFN- γ .

individual consultations, and managing teams to prevent and treat common respiratory illnesses that can impair training and competitive performance. This study was undertaken to develop a combined clinical and laboratory-based paradigm to identify athletes at higher risk of respiratory illness associated with respiratory infections and/or inflammation. The three key elements of the predictive model of increased risk of upper respiratory tract illness were low levels of IgA in pre exercise saliva samples, detection of viral EBV DNA in saliva, and a genetic predisposition for pro-inflammatory cytokine responses.

There was a higher prevalence of low levels of salivary IgA in the illness-prone athletes but the group median concentrations were not significantly different between the groups. This outcome highlights the need for monitoring of changes in salivary IgA in individual athletes rather than cohort means or medians. Although salivary IgA is a promising biomarker (58) it exhibits substantial within- and between-subject variability (13). There was also a trend for higher detection rates of EBV-DNA in saliva of the illness-prone group. This is consistent with previous studies of high detection rates of EBV-DNA prior to the appearance of URS in illness-prone athletes with low levels of salivary IgA (28).

There was insufficient power to examine the potential risk of the cytokine response to the standardised exercise protocols (data not shown) as the technology used proved inadequate for the detection of many of the low levels of plasma cytokines. However, a predisposition to pro-inflammatory responses has been identified in previous studies of long-distance runners (11) and these responses need to be re-examined in other sporting disciplines. The resting and post-exercise levels of C-reactive protein did not correlate with infection risk and supports previous findings (10) that C-reactive protein is not a good biomarker for identifying athletes at risk of URS.

The cytokine polymorphisms indicated a possible association with higher prevalence of the minor alleles for IL-6 and IFN- γ in the illness-prone athletes and an increasing risk score with the total number of minor cytokine alleles overall. These patterns support previous studies (5) and indicate athletes with a genetic predisposition to pro-inflammatory cytokines responses or impaired anti-inflammatory response (5, 61) may have a higher risk of URS.

The response of midkine to the two exercise protocols showed a rapid substantial increase

Table 9. Number (and percentage) of minor alleles in the healthy and illness-prone athletes for each cytokine, mean and the composite genetic risk score for minor alleles and difference between groups (mean (SD)). Comparisons between groups with significant differences or trends are indicated *.

Cytokine	SNP ID	Number of minor alleles (Risk Score)	Healthy (n=12)	Illness prone (n=4)	Total population	p-value
IL-4	rs2246250	0	8 73%	3 27%	11 69%	0.76
		1	4 80%	1 20%	5 31%	
		Genetic risk	0.3 (0.5)	0.3 (0.5)	0.3 (0.5)	
IL-6	rs1800795	0	3 50%	3 50%	6 38%	0.15
		1	4 80%	1 20%	5 31%	
		2	5 100%	5 31%	5 31%	
IL-8	rs4073	0	4 80%	1 20%	5 31%	0.40
		1	6 86%	1 14%	7 44%	
		2	2 50%	2 50%	4 25%	
IL-10	rs1800896	0	8 67%	4 33%	12 75%	0.18
		1	4 100%	4 25%	4 25%	
		Genetic risk	0.3 (0.5)	0.0 (0.0)	0.3 (0.4)	
IL-17a	rs2275913	0	5 71%	2 29%	7 44%	0.83
		1	6 75%	2 25%	8 50%	
		2	1 100%	1 6.3%	1 6.3%	
IL-17a	rs1974226	0	7 64%	4 36%	11 69%	0.30
		1	4 100%	4 25%	4 25%	
		2	1 100%	1 6.3%	1 6.3%	
IL-1ra	rs419598	0	5 63%	3 38%	8 50%	0.49
		1	6 86%	1 14%	7 44%	
		2	1 100%	1 6.3%	1 6.3%	
IFN-γ	rs2430561	0	1 25%	3 75%	4 25%	0.03*
		1	9 90%	1 10%	10 63%	
		2	2 100%	2 50%	2 13%	
Minor alleles	Genetic risk score	Mean (SD)	5.6 (2.1)	2.8 (1.75)	4.9 (2.3)	0.03*

immediately after exercise that returned to baseline concentrations within 24 h post-exercise. The changes in midkine concentrations most likely reflect responses to the renin-angiotensin pathways that control midkine activity (42). There were no significant differences in the responses of the illness-prone and healthy athletes, and therefore the midkine responses do not provide additional information to a paradigm for risk of URS. The marked elevation in midkine concentration after exercise probably reflects short-term mobilisation of the pro-

Table 10: Increased risk of URS for each individual SNP minor alleles and the composite genetic risk score for the cumulative addition of minor alleles. Univariate Odds Ratios (OR) and 95% confidence intervals (CI) derived from exact logistic regression.

Cytokine	SNP ID (NCBI)	OR (95% CI) for 3 or more URS episodes	p-value
IL-4	rs2246250	0.68 (0.01, 12.4)	1.00
IL-6	rs1800795	0.18 (0.00, 1.34)	0.13
IL-8	rs4073	2.06 (0.36, 16.7)	0.58
IL-10	rs1800896	0.46 (0.00, 3.37)	0.54
IL-17a	rs2275913	0.64 (0.04, 6.11)	1.00
IL-17a	rs1974226	0.37 (0.00, 1.96)	0.36
IL-1ra	rs419598	0.27 (0.00, 3.12)	0.51
IFN-γ	rs2430561	0.05 (0.00, 0.96)	0.05
Minor allele score	Genetic risk score	0.49 (0.15, 0.98)	0.04

tein sequestered in tissue such as endothelial cells lining blood vessels rather than endogenous synthesis of midkine (42).

The clinical assessments were unremarkable, and the only relevant finding was a tendency towards a higher prevalence of a combined history of asthma and allergy to inhaled allergens in the athletes with a lower incidence of URS, although the numbers were small. The records of current medications indicated that these athletes were well managed therapeutically for medical conditions suggesting the lower URS incidence may be the consequence of good clinical management. Knowing the EBV serology status was important for interpreting the EBV-DNA data but otherwise there were no pathology tests of significance to the risk paradigm. The history of URS in the 12 months prior to the study did not correlate with the prospective data recorded during the study and confirmed by the sports physician, suggesting a self-reported history of URTI may not be a good indicator of potential future risk of URS.

We have taken the outcomes of this study, combined them with previous published data, to formulate a framework for categorising underlying factors associated with recurrent URS as uncontrollable, controllable, or partially controllable risks (Table 12). This table provides a checklist for clinicians and trainers working with highly trained athletes, and researchers investigating the causes, diagnosis, treatment and management strategies for common respiratory illnesses experienced by athletes.

Uncontrollable risks

The uncontrollable risks include genetic risks associated with an individual's predisposition to a pro-inflammatory response. Inherited cytokine polymorphisms will influence the cytokine response to infections and other inflammatory stimuli. Characterising the underlying genetic risk may be beneficial for identifying an athlete at risk of pro-inflammatory responses. Despite the small cohort size in this study, the genetic risk scores from the cumulative addition of minor alleles for each cytokine SNP provided some indication of an increased risk of URS with a higher number of minor alleles. Significantly larger cohort studies are required to confirm this outcome. However the outcomes are consistent with previous studies examining individual cytokine gene polymorphisms (5), suggesting the high-expression genotype for IL-6 may be associated with an increased likelihood of >3 URS/year. The study also indicated a possible association with the IFN-γ genotype which is associated with increased severity of illness symptoms (57). A defect in IFN-γ secretion has also been identified in athletes presenting with persistent fatigue and impaired performance (3).

As the cytokine responses to exercise are determined by the genotype these could also be classified as uncontrollable risks. In this investigation there was insufficient data and power to reliably assess the cytokine responses by the cyclists to the two exercise protocols. A previous study of long-distance runners identified impaired inflammatory regulation in illness-prone athletes (11), with higher levels of IL-6 and lower levels of IL-10 and IL-1ra after intense exercise. Further studies are required to determine if these cytokine responses to a standardised exercise test can be included in a risk assessment paradigm. Genetic differences in IL-10 SNPs

Table 11. Midkine concentration (pg/mL) pre-exercise and the post-exercise change for all athletes undertaking the Intense and Long Exercise Protocols at each collection time (Figure 3).

Time point	Intense Protocol			Long Protocol		
	Median (95% CI)	Change (95% CI)	<i>p</i> value	Median (95% CI)	Change (95% CI)	<i>p</i> value
Pre-exercise	750 (460, 958)	257 (+220, +400)	<0.0001	590 (+513, +865)	359 (+247, +505)	<0.0001
Post-exercise	1007 (681, 1237)	-289 (-414, -188)	0.0001	969 (+665, +1294)	-272 (-318, -174)	<0.0001
1 h Post	614 (435, 917)	-342 (-480, -212)	0.0002	716 (+563, +912)	-345 (-483, -177)	<0.001
24 h Post	648 (358, 851)	-89 (-162, +188)	0.32	600 (+475, +999)	-11 (-112, +117)	0.89

Table 12. Controllable and Uncontrollable risk factors for upper respiratory symptoms in athletes.

Risk and Category	Description of Risk for URS	References	
Uncontrollable Risks			
Genetic risk	High expression IL-6 genotype	Cox <i>et al</i> (2010) (5) Current study	
	Low-expression IL-2 genotype	Cox <i>et al</i> (2010) (5)	
	High expression IL-10 genotype	Zehsaz <i>et al</i> (2014) (61)	
	Low expression for IFN- γ (minor alleles)	Current study	
	Genetic risk score for cumulative number of minor cytokine alleles	Current study	
	Gender	He <i>et al</i> (2014) (30)	
Immune responses to exercise	Low resting IL-8, IL-10, IL-1ra levels	Cox <i>et al</i> (2007) (11)	
	Low post-exercise IL-10, IL-1ra	Cox <i>et al</i> (2007) (11)	
	High post-exercise IL-6	Cox <i>et al</i> (2007) (11)	
	Post-exercise IL-10 production	Gleeson <i>et al</i> (2011) (21)	
Controllable Risks			
Health Risks	Asthma	Reid <i>et al</i> (2004) (49) Cox <i>et al</i> (2008) (9)	
	Allergy to inhaled allergens	Schwellnus <i>et al</i> (1997) (51) Katelaris <i>et al</i> (2000) (34) Katelaris <i>et al</i> (2003) (35) Cox <i>et al</i> (2010) (7)	
	Immunodeficiency and autoimmune states	Fricke <i>et al</i> (1999) (16) Reid <i>et al</i> (2004) (49)	
	Vaccinations	Gärtner and Meyer (2014) (18)	
	Hygiene	Pyne <i>et al</i> (2000) (47)	
	Nutritional status	Gleeson & Pyne (2015) (27)	
	Low Vitamin D	He <i>et al</i> (2013) (31)	
	EBV status and viral reactivation	Gleeson <i>et al</i> (2002) (28) Cox <i>et al</i> (2004) (8)	
	Psychological stress	Konig <i>et al</i> (2000) (38)	
	Sleep disturbance	Konig <i>et al</i> (2000) (38)	
	Partially Controllable Risks		
	Environmental Risks	Winter season	Konig <i>et al</i> (2000) (38) Hellard <i>et al</i> (2015) (32) Svendsen <i>et al</i> (2016) (55)
		Cold air exposure	Passali <i>et al</i> (2004) (45)
Extreme temperatures (hot/cold)		Walsh <i>et al</i> (2011) (58)	
Exposure to infections		Pyne <i>et al</i> (2000) (47)	
International air travel		Svendsen <i>et al</i> (2016) (55)	
Training and Competition	Respiratory epithelial damage due to airway drying or trauma	Passali <i>et al</i> (2004) (45) Martin <i>et al</i> (2012) (40)	
	Circadian rhythm disturbance	Reilly <i>et al</i> (2007) (50)	
	Low salivary IgA levels and excretion rates	Gleeson <i>et al</i> (1999) (26) Walsh <i>et al</i> (2011) (58) Neville <i>et al</i> (2008) (43) Ihalainen <i>et al</i> (2016) (33)	
	Low tear fluid SIgA levels	Hanstock <i>et al</i> (2016) (29)	
	Training monotony	Svendsen <i>et al</i> (2016) (55)	

have been associated with susceptibility to URS due to impaired IL-10 responses (5, 61), making IL-10 a candidate cytokine gene for further investigation.

Controllable risks

A comprehensive clinical assessment can assist with identifying and managing clinical conditions associated with URS. Ensuring appropriate therapeutic control of asthma and allergy to inhaled allergens can reduce the risk of upper respiratory illness and associated fatigue (1, 35, 49). The suggestion of a higher incidence of allergy in the athletes with a lower incidence of illness in this study may indicate these conditions were well controlled in these athletes, as the majority had rhino-conjunctivitis and recorded use of relevant therapeutics. Other controllable underlying health risks include ensuring adequate levels of Vitamin D (31) and management of psychological stress (38).

Determining the EBV serology status assists with managing athletes. Those athletes who are seronegative can be advised and managed to avoid primary infection. Seropositive athletes can be monitored for viral reactivation, such as expression of EBV-DNA in saliva. This study identified a higher expression rate of EBV-DNA in the illness-prone athletes. It is unlikely that a therapeutic intervention would be implemented but the EBV can be controlled with anti-viral therapy (8). Detection of EBV-DNA is not routinely available but this study confirmed previous reports of lower salivary IgA levels in illness-prone athletes prior to the detection of EBV viral reactivation (28) and the availability of point-of-care salivary IgA tests (4) make this a useful biomarker. However, as highlighted in this study, EBV seronegative subjects can still be at risk of recurrent URS from other causes (Table 2). Even with the small number of subjects in this study, there was a trend for an association of low levels of salivary IgA with an increase in illness (Table 5). Monitoring tear fluid (29) or salivary IgA (4, 23, 33, 43) can assist in modifying training regimes to limit the impact of upper respiratory symptoms associated with immune activation/inflammation on training and competitive performance.

Partially controllable risks

While the season cannot be controlled, knowing there is an increased risk for URS associated with training and competing during the winter months (32, 38) can assist

with modifying training and implementing personal avoidance strategies for at-risk athletes. The impact of cold air on mucosal membranes has not been extensively studied but is known to increase the symptoms that mimic respiratory infections (36). Similarly, risks of some infections can be reduced by prior vaccination (18), reducing exposure to potential pathogens and implementing personal hygiene strategies (47).

The Challenges

The challenges facing researchers involved in studies of high-performance athletes include the selection of appropriate performance and laboratory tests, limitations of some analytical methods, self-reported clinical data, sample size estimation and recruitment of sufficient subjects, and statistical analyses in small sample sizes to provide meaningful outcomes. This study experienced all these challenges. The ability to recruit an adequate sample size that had the statistical power proved a challenge for this complex study design, particularly given the extended study period. In this study the requirement for daily reporting over a 9 month period impacted negatively on the recruitment process. In turn, this limitation impacted on the statistical power of the study outcomes. Based on the most commonly measured parameter and assuming a mean difference in salivary IgA concentration of ~10-15 mg/L and a standard deviation of ~10 mg/L in a parallel groups design, a minimum of 15 subjects would be needed in both healthy and illness-prone groups.

This study was costly, not only for the exercise and laboratory testing components, but labour intensive, as it involved exercise physiologists, sports physicians and multiple experienced laboratory scientists, as well as web designers for the 9 month prospective on-line reporting, and statisticians. To overcome one of the criticisms of investigations of self-reported upper respiratory illness by elite athletes, this study included physician verification of the symptoms for each reported episode. This added to the commitment by the athletes and the sports physicians but was deemed important for collection of higher order clinical data. Additional pathology testing, such as determination of IgE-specific antibodies for aero-allergens, infectious serology and pathogen identification would have been informative but expensive.

An unexpected challenge was the selection of the analyser for the simultaneous measurement of the multiple cytokines. The analyser proved unsuitable for the assessment of the plasma cytokine responses to the exercise protocols, with most cytokine levels being below the limit of detection of the assay, highlighting the need to ensure any new technology is appropriate for the study sampling regime. The lack of cytokine responses limited the interpretation of the cytokine genotypes and relationships to URS in this cohort.

CONCLUSIONS

Paradigms for assessing the risk of respiratory illness in high-performance athletes historically have not been well defined. High performance athletes experiencing recurrent respiratory illness should be assessed clinically and monitored to eliminate or reduce controllable risks. The laboratory parameters

identified with an increased risk of illnesses included cytokine gene polymorphisms for the high expression of IL-6 and IFN- γ , expression of EBV-DNA in saliva, and low levels of salivary IgA. SNP analysis is not routinely performed and monitoring viral reactivation is usually confined to research settings. Recent development of point-of-care analysers for salivary IgA allows for real-time assessment of the risk of URS in individual athletes and may prove beneficial for adoption of preventive strategies.

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